New insights into the peroxisomal protein inventory: Acyl-CoA oxidases and –dehydrogenases are an ancient feature of peroxisomes

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Abbreviations: ACAD, acyl-CoA dehydrogenase, ACOX, acyl-CoA oxidase, FAD, flavin adenine dinucleotide; MTS, mitochondrial targeting signal; Pex, peroxin; PMP, peroxisomal membrane protein; PTS, peroxisomal targeting signal; ROS, reactive oxygen species

Abstract

Peroxisomes are ubiquitous organelles which participate in a variety of essential biochemical pathways. An intimate interrelationship between peroxisomes and mitochondria is emerging in mammals, where both organelles cooperate in fatty acid β-oxidation and cellular lipid homeostasis. As mitochondrial fatty acid β-oxidation is lacking in yeast and plants, suitable genetically accessible model systems to study this interrelationship are scarce. Here, we propose the filamentous fungus *Ustilago maydis* as a suitable model for those studies. We combined molecular cell biology, bioinformatics and phylogenetic analyses and provide the first comprehensive inventory of *U. maydis* peroxisomal proteins and pathways. Studies with a peroxisome-deficient Δ*pex3* mutant revealed the existence of parallel and complex, cooperative β-oxidation pathways in peroxisomes and mitochondria, mimicking the situation in mammals. Furthermore, we provide evidence that acyl-CoA dehydrogenases (ACADs) are *bona fide* peroxisomal proteins in fungi and mammals and together with acyl-CoA oxidases (ACOX) belong to the basic enzymatic repertoire of peroxisomes. A genome comparison with baker's yeast and human gained new insights into the basic peroxisomal protein inventory shared by humans and fungi and revealed novel peroxisomal proteins and functions in *U. maydis*. The importance of our findings for the evolution and function of the complex interrelationship between peroxisomes and mitochondria in fatty acid β-oxidation is discussed.

1. Introduction

Peroxisomes are single membrane-bound organelles which are ubiquitously found in eukaryotic cells. They are highly dynamic with large plasticity and display an unprecedented versatility in their functions [1]. Typical functions of peroxisomes are the β-oxidation of fatty acids and the metabolism of hydrogen peroxide. Other important reactions in humans include fatty acid α-oxidation, degradation of D-amino acids, catabolism of purines, and biosynthesis of ether lipids, polyunsaturated fatty acids and bile acids [2]. Peroxisomal dysfunctions have been linked to inherited disorders that are associated with multiple severe clinical symptoms [3], but also to ageing and age-related diseases [4]. Moreover, several novel and often unexpected peroxisome functions have been identified including roles in signalling by reactive oxygen species (ROS) and fine tuning of cellular functions or antiviral innate immunity in man [5, 6].

In this respect, a surprisingly close interrelationship between peroxisomes and mitochondria, the "peroxisome-mitochondrion connection" has been revealed [7-10]. The two organelles cooperate in β-oxidation of fatty acids (FA) in animals, whereas in plants and yeast this pathway is exclusively peroxisomal [11]. Both organelles contain their specific set of β-oxidation enzymes, which catalyse similar reactions. The peroxisomal β-oxidation pathway starts with an acyl-CoA oxidase (ACOX) transferring electrons in the form of H^- from their prosthetic group $FADH_2$ to O_2 thereby generating hydrogen peroxide, which is detoxified by catalase. In contrast, mitochondrial β-oxidation relies on acyl-CoA dehydrogenases (ACAD), which transfer electrons to FAD thereby generating $FADH_2$ which is used for ATP production in the respiratory chain. Moreover, both pathways diverge in their substrate specificities: in mammals, peroxisomes preferentially oxidise saturated and unsaturated very long-, long- and branched-chain fatty acids, whereas mitochondria more efficiently oxidise long-, middle- and short-chain fatty acids. In addition, peroxisomes only chain-shorten fatty acids, which are

subsequently conjugated to carnitine and routed to mitochondria for complete oxidation. This interconnection between peroxisomal and mitochondrial fatty acid breakdown requires a coordinated regulation of the lipid metabolism in both organelles, as well as metabolite transfer among them – a process which is still poorly understood.

Besides a metabolic cooperation in fatty acid β-oxidation, peroxisomes and mitochondria contribute to cellular ROS homeostasis and share a redox-sensitive relationship [12, 13]. Strikingly, they also share key proteins of their division machinery which is indicative of a coordinated biogenesis under certain conditions and requires organised targeting and recruitment of those proteins [14, 15]. Furthermore, the two organelles cooperate in anti-viral signalling and defence [6], and a vesicular trafficking pathway from mitochondria to peroxisomes has been described [16]. Based on these findings, we proposed the medically relevant concept of the "peroxisome-mitochondrion connection" suggesting that peroxisomal alterations in metabolism, biogenesis, dynamics and proliferation can potentially influence mitochondrial functions, and *vice versa* [7, 10]. As such inter-organelle dependencies can activate a cascade of cellular events with pathological consequences, there is great interest in improving our understanding of organelle cooperation and interplay in cell physiology and disease [10].

The use of fungal model systems such as the budding yeast *Saccharomyces cerevisiae* has contributed enormously to our understanding of fundamental cellular and disease-related processes in animals and humans. Studies on organelle cooperation and interplay are, however, limited, as certain processes found in animal cells (e.g., cooperation of peroxisomes and mitochondria in fatty acid degradation and microtubule-based organelle transport) do not exist in budding yeast. The basidiomycete *Ustilago maydis* has recently been introduced as a new model for cell biological studies [17] as it provides the technical advantages of unicellular organisms and shares many important processes with human cells such as long-distance transport along microtubules, polarized growth, and open mitosis). A genome-wide

comparison of the predicted proteomes of *U. maydis*, *S. cerevisiae* and *H. sapiens* revealed that *U. maydis* and humans share a significant number of proteins that are not found in *S. cerevisiae* [18]. More importantly, several of the shared proteins have been related to serious human diseases and a large portion is of unknown function.

In this study, we combined molecular cell biology, bioinformatics and phylogenetic analyses and provide the first comprehensive inventory of *U. maydis* peroxisomal proteins and pathways. A genomic comparison with other organisms such as *S. cerevisiae* and *H. sapiens* gained new insights into the basic peroxisomal protein inventory shared by humans and fungi and revealed novel peroxisomal proteins and functions in *U. maydis*. We demonstrate that *U. maydis* possesses a complex and complete enzymatic inventory for both peroxisomal and mitochondrial fatty acid β-oxidation, which renders *U. maydis* a suitable model system to study the metabolic cooperation and interplay of both organelles. Furthermore, we provide evidence that ACADs are *bona fide* peroxisomal proteins in fungi and mammals and belong to the basic enzymatic repertoire of peroxisomes. The importance of our findings for the evolution and function of the complex interrelationship between peroxisomes and mitochondria in fatty acid β-oxidation is discussed.

2. Materials and Methods

2.1 Search for potential peroxisomal genes in *U. maydis*

The *Ustilago maydis* 521 genome database (http://pedant.gsf.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi) was screened for proteins carrying a peroxisomal targeting signal 1 (PTS1) at the very C-terminus using the consensus (ASCNPHTG)-(RKHQNS)-(LMIVF) [19]. Thereafter, the identified proteins were further analysed by PTS1 predictor algorithms [20] and sequences which produced no hit with the "general" modus of the software were removed. For the remaining candidates a TMD prediction was performed using the TMHMM software; proteins with more than one predicted TMD were not considered to bear a functional PTS1. For further validation all remaining sequences were screened for conservation of the potential PTS1 using BLAST2.0. Sequences, which already lost the potential PTS1 among Ustilaginomycetes were rated as likely false positives and were removed from the lists. Additionally, mitochondrial targeting was examined using Mitoprot2 [21], and Predotar1.03 [22]; potential targeting to the secretory pathway was screened with TargetP1.1 [23]. *Peroxisomal* targeting signal 2 (PTS2) was analysed by PTS2 prediction algorithms [24]. Functions were attributed to the potential peroxisomal proteins with regard to their homology to known proteins from other species and proteins were organised into specific metabolic pathways. The MIPS *Ustilago maydis* database (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/) was screened for peroxisomal proteins without a PTS1, namely peroxisomal membrane proteins and peroxins, by key word search and BLAST analysis. Furthermore, published data was included [25]. To validate the homology of *U. maydis* peroxisomal proteins to mammalian counterparts, phylogenetic trees were constructed. Related sequences for the main peroxisomal and mitochondrial β-oxidation proteins from ≥ 30 fungal and animal species were searched via reciprocal BLAST analysis and aligned with

ClustalW. The multifunctional enzymes found in the second and third step of peroxisomal and mitochondrial β-oxidation were separated into their correspondent enoyl-CoA and dehydrogenase domains for proper alignment. Correct alignment of all sequences was checked and if necessary corrected manually using the BioEdit software. Phylogenetic reconstructions were done with PhyML3.0 using the aLRT algorithm for branch support and including NNI and SPR tree searching operations. Multiple rounds of phylogenetic tree construction were used to identify outliers which were subsequently removed from the tree.

2.2 Plasmids and strains generated for studies in *U. maydis*

To visualise peroxisomes in *U. maydis*, a mCherry-SKL fusion construct was generated. The *gfp* and the carboxin resistance cassette of plasmid poGFP-SKL [26] were replaced with mCherry and a hygromycin resistance cassette resulting in plasmid pomChSKL. The plasmid was linearised with *Eco*RV and integrated ectopically into the strain FB1 resulting in FB1mChSKL. To visualize acyl-CoA oxidase (ACOX, um02208), acyl-CoA dehydrogenase 11n (ACAD11n, um06400), acyl-CoA dehydrogenase medium-chain (ACADM, um01049), acyl-CoA dehydrogenase short-branched (ACDSB, um06185), acyl-CoA dehydrogenase 11c (ACAD11c, um06422), lactamase beta 2 (LACTB, um11901) and catalase (CAT, um11067) in *U. maydis*, correspondent poGFP- or mCH-constructs were generated through *in vivo* recombination in the *Saccharomyces cerevisiae* strain DS94 (MATα, *ura3-52*, *trp1-1*, *leu2-3*, *his3-111*, and *lys2-801* [27] following published procedures [28]. For all the recombination events, the fragments were amplified with 30 bp homologous sequences upstream and downstream of the sequences of interest and cloned individually into vector pNEBhyg-yeast [29] along with 878 bp *Otef* promoter, 717 bp *gfp* or 711 bp *mCherry* and 307 bp *Tnos* terminator. ACOX and ACAD11n plasmids were digested with *Nde*I and integrated ectopically into the peroxisomal marker strain FB1mChSKL resulting in FB1mChSKL_GFPACOX and FB1mChSKL_GFPACAD11n, respectively. ACADM and

ACDSB plasmids were digested with *Xmn*I and integrated ectopically into the mitochondrial marker strain FB2LgaGFP [26] resulting in FB2LgaGFP_ACADMmCh and FB2LgaGFP_ACDSBmCh, respectively. ACAD11c and LACTB plasmids were digested with *Dra*I and integrated ectopically into the mitochondrial marker strain FB2LgaGFP [26] and peroxisomal marker strain AB33GFPSKL resulting in FB2LgaGFP_mChACAD11, FB2LgaGFP_mChLACTB, AB33GSKL_mChACAD11, and AB33GSKL_mChLACTB, respectively. CAT plasmid was digested with *Dra*I and integrated ectopically into the peroxisomal marker strain AB33GFPSKL resulting in AB33GSKL_mChCAT. For deletion of *pex3* in *U. maydis*, plasmid p∆*pex3* was generated through *in vivo* recombination (see above). The 1847 bp *pex3* (um06200) gene was amplified with 30 bp overhangs by PCR and cloned into cloning vector pNEBhyg-yeast [29] along with 1030 bp *NudE* promoter and 1158 bp *pex3* terminator resulting in p∆*pex3.*The plasmid p∆*pex3* was digested with *Dra*I and integrated homologously into the *pex3* locus of the strain Sg200, resulting in Sg200 ∆*pex3*. For a list of strains and primers see **Suppl. Tables S1 and S2**.

2.3 Laser-based epifluorescence microscopy

Microscopy of *U. maydis* was performed as previously described[29]. Briefly, cells were placed on a thin layer of 2% agarose, covered with a cover slip, and immediately observed using a IX81 motorized inverted microscope (Olympus, Hamburg, Germany) and a VS-LMS4 Laser-Merge-System equipped with 488 and 561 nm, 75mW solid-state lasers using Dual-View Microimager (Optical Insights, Tucson, USA) and appropriate filters. LgaGFP, GFP-SKL, GFP-ACOX and GFP-ACAD11n were visualised at 150ms exposure time at 10% of the 488 nm laser; ACADM-mCh, ACDSB-mCh, mCh-ACAD11c, mCh-LACTB, and mCh-CAT were visualised at 150ms exposure time at 50% of the 561 nm laser. Images were captured using a charged-coupled device camera (Photometric CoolSNAP HQ2, Roper Scientific,

Germany). All parts of the system were under the control of the software package MetaMorph (Molecular Devices, Downingtown), which was also used for image processing and overlay.

2.4 Scanning electron microscopy

For scanning electron microscopy, the wild type (Sg200) and the deletion strain (Sg200Δ*pex3*) were grown overnight in complete medium (CM) [30] containing 1% glucose at 28ºC and 200 rpm until an OD of 0.6-0.8. Cells were then plated on CM-glucose-charcoal plates and incubated for 2-3 days at 28ºC. Subsequently, the samples were attached to a cryo-sledge and rapidly frozen in liquid nitrogen, followed by water sublimation at 295uC for 3 min using the Alto 2100 chamber (Gatan Ltd., Oxfordshire,UK). This was followed by gold sputtering and observation in a JeolJSM-6390LV scanning electron microscope (JEOL, Ltd., WelwynGarden City, UK)

2.5 Induction of peroxisome proliferation by oleic acid

FB2 GFP-SKL cells were grown overnight in CM containing 1% (w/v) glucose at 28°C and 200 rpm, until an OD_{600} of 0.8. Cells were centrifuged at 3000 rpm for 10 min and washed twice with nitrogen minimal medium (NM) to remove all carbon sources. The pellet was resuspended in NM medium and equal volumes were transferred into flasks with NM medium containing 1% (w/v) glucose (control) or 0.2% (v/v) oleic acid (Merck) (final concentration) and incubated for 1hr at 28ºC and 200 rpm. Peroxisome numbers were determined by laser-based epifluorescence microscopy. Z-stacks were taken at 300 nm step size with 150 ms exposure time using a Piezo drive (Piezosystem Jena GmbH, Jena, Germany) and analysed as maximum projection using MetaMorph.

To determine induction of ACOX, total RNA was extracted according to [31], and DNAse treated (DNA free kit, Ambion). cDNA was synthesised by reverse transcriptase PCR using the Superscript III First-Strand Synthesis Super mix kit (Invitrogen). Parts of the coding

sequences of ACOX (um02208), ACADM (um01049), ACDSB (um06185), Elongation Factor 1 (EF1; um00924) and peptidyl-prolyl cis–trans isomerase (PPI, um03726) (the latter two used as internal control), were subsequently amplified by PCR using gene-specific primers (**Suppl. Table S2**). Samples were separated on 1% agarose gels and analysed using the Alpha Imager HP (Alpha Innotech, San Leandro, USA).

2.6 Growth assays on fatty acids

To examine growth on different fatty acids, wild type cells (SG200) and those lacking peroxisomes (SG200Δ*pex3*) were grown overnight in CM containing 1% glucose at 28ºC and 200 rpm until an OD of 0.6-0.8. Cells were washed 3 times and resuspended in 5 ml of sterile NM. A 5% dilution was plated on NM-Agar plates supplemented with different fatty acids, acetate or glucose. The following fatty acids (from Sigma-Aldrich, St. Louis, MO if not stated otherwise) (0.001%) were used: butyric acid (4:0), valeric acid (5:0), hexanoic acid (6:0), decanoic acid (10:0), myristic acid (14:0), myristoleic acid (14:1(n-5)), palmitic acid (16:0), palmitoleic acid (16:1(n-7)), oleic acid (18:1(n-9)) (Merck, Darmstadt, Germany), pristanic acid (2,6,10,14-tetramethyl 15:0), phytanic acid (3,7,11,15-tetramethyl 16:0), erucic acid (22:1(n-9)) and lignoceric acid (24:0). Fatty acids were dissolved in ethanol (100 mg/mL stock solutions, final ethanol concentration: 0.01%). Glucose and acetate were used at 1%. Plates were incubated for 3 days at 28ºC and images acquired using stereomicroscope Nikon SM2 800, camera control pro 2.7.1 (Nikon®). The assays were repeated at least 3 times.

2.7 cDNAs and antibodies for studies in mammalian cells

N-terminally Myc-tagged expression constructs for human ACAD11 (Myc-*Hs*ACAD11) and *U. maydis* ACAD11n (Myc-*Um*ACAD11n) were created as follows: human ACAD11 (NM_032169.4) was amplified from HepG2 cDNA, and *U. maydis* ACAD11n from *U. maydis* 521 genomic DNA (**Suppl. Table S2**). Rat β-Lactamase (β-Lactb2;

NM_001024247.1) was amplified from a TOPOII construct [32]. All three PCR products were inserted into pCMV-TAG3A vectors (Agilent Technologies, La Jolla, CA, USA). The Myc-tagged expression construct for rat ACAD11 (Myc-*Rn*ACAD11) is described elsewhere [32]. C-terminally tagged constructs for rat (*Rn*ACAD11-Myc), *U. maydis* (*Um*ACAD11n-Myc) and human (*Hs*ACAD11-Myc) ACAD11 were generated from the previous plasmids (**Suppl. Table S2**). PCR products were inserted into vector pCMV-TAG5b. In-frame insertion of all constructs was verified by sequencing (Eurofins MWG, Ebersberg, Germany).

Antibodies used in this study were kind gifts from D. Crane, Griffith University, Brisbane, Australia (Pex14), T. Hashimoto, Shinshu University School of Medicine, Nagano, Japan (ACOX1), or purchased from Abcam, Cambridge, UK (ACAD11, ERp29, VDAC1, Ornithine transcarbamoylase), BD Biosciences, Franklin Lakes, USA (GRP78, ATP synthase α), Cell Signaling Technology, Danvers, USA (α/β tubulin) and Santa Cruz Biotechnology, Santa Cruz, USA (Myc epitope 9E10). Species-specific anti-IgG antibodies conjugated to the fluorophores TRITC, Alexa 488 or horseradish peroxidase were obtained from Jackson ImmunoResearch (West Grove, USA) and Invitrogen (Life Technologies, Grand Island, NY, USA).

2.8 Mammalian cell culture, transfection, microscopy and subcellular fractionation

COS-7 (ATCC CRL-1651), HeLa (ATCC CCL-2) and HepG2 cells (ATCC HB8065) were maintained in DMEM supplemented with 10% FCS (PAA Laboratories GmbH, Cölbe, Germany) at 37ºC in a 5% CO_2-humidified incubator. Cells were transfected with DNA constructs by incubation with polyethylenimine (25 kDa PEI, Sigma-Aldrich, St. Louis, MO, USA) as described [33] or with Turbofect (Fermentas) according to the manufacturer's instructions. For immunofluorescence microscopy, cells grown on glass coverslips were fixed with 4% para-formaldehyde in PBS, pH 7.4, permeabilised with 0.2% Triton X-100, blocked with 1% BSA and incubated with primary and secondary antibodies as described [34].

Microscopy analysis was performed using an Olympus IX81 microscope (Olympus Optical, Hamburg, Germany) equipped with a PlanApo 100x/1.40 oil objective and filter sets 41020 and 41004 (Chroma, Bellows Falls, USA). Images were acquired with an F-view II CCD camera (Soft Imaging System, Munster, Germany) driven by Soft Imaging software. Confocal images were obtained using a Zeiss LSM 510 Meta confocal setup (Carl Zeiss, Oberkochen, Germany) equipped with Plan-Apochromat 100x/1.4 oil objectives. Images were processed using LSM 510 software (Carl Zeiss MicroImaging, Inc.). Background noise was minimal when optimal gain/offset settings for the detectors were used. Peroxisome isolation from rat liver was performed as described [35].

3. RESULTS

3.1 ACADs belong to the basic repertoire of peroxisomal enzymes

Fatty acid β-oxidation is exclusively peroxisomal in yeast and plants, but is distributed between peroxisomes and mitochondria in animals. Mitochondrial β-oxidation relies on acyl-CoA dehydrogenases (ACADs). Whereas humans encode 11 members of the ACAD protein family, they are completely absent in the yeast *S. cerevisiae* [36] (**Fig. 1; Suppl. Fig. S1**). We initially focused our studies on the detection and characterization of ACADs in *U. maydis*, as their presence is a prerequisite for mitochondrial fatty acid β-oxidation, and thus potential metabolic cooperation in peroxisomal and mitochondrial lipid metabolism. BLAST searches revealed that *U. maydis* encodes seven putative ACADs; two of which are predicted to be targeted to mitochondria. Surprisingly, we also identified four ACADs (um10665, um01466, um06400 and um00122) with a predicted PTS1 suggesting peroxisomal targeting (**Table 1**). Our phylogenetic analyses revealed that orthologues of those genes exist throughout fungi and even among animals (um00122), but only um06400 was found across all species investigated (**Fig. 1; Suppl. Fig. S1**). *U. maydis* um06400 represents a homolog of the mammalian ACAD11 (**Table 1; Suppl. Table S3).** All representatives of this enzyme subfamily end with

a C-terminal PTS1 indicating a phylogenetically conserved contribution to peroxisomal β-oxidation (**Suppl. Fig. S1**). Interestingly, the fungal version (ACAD11n) lacks the frontal aminoglycoside phosphotransferase (APH) domain found among animals [37] (**Fig. 1**). However, we identified individual proteins showing homology to this phosphotransferase domain throughout all fungi including *U. maydis* (um06422, named ACAD11c in this study) which, strikingly, share putative PTS1 sequences (**Suppl. Fig. S1**). This implies that in animals both ACAD and APH domains have been combined in one protein to fulfil proper ACAD11 function (**Fig. 1**).

To verify the unusual peroxisomal localization of ACADs, which are generally attributed to mitochondrial β-oxidation, we generated a GFP-tagged variant of um06400 (ACAD11n), which is conserved among all opisthokont organisms. Furthermore, we generated an N-terminal mCherry fusion of um06422 representing the putative aminoglycoside phosphotransferase (APH) domain typically found in animal ACAD11 (mCherry-ACAD11c). We used EGFP or mCherry fused to the PTS1 SKL as a peroxisomal marker in *U. maydis* [26]. Expression of GFP-ACAD11n in an *U. maydis* mCherry-SKL strain revealed a clear co-localisation, thus confirming that *U. maydis* ACAD11n is a *bona fide* peroxisomal protein (**Fig. 2A-C**). In addition, mCherry-ACAD11c (APH) was properly targeted to peroxisomes in GFP-SKL expressing cells (**Fig. 1D-F**). This confirms that in *U. maydis* both individual proteins are routed to peroxisomes, where they can potentially cooperate to fulfil ACAD11 function.

The homologous mammalian ACAD11 was first identified in purified peroxisomes from rat liver in several proteomics approaches [32, 38, 39]. A peroxisomal localisation has, however, been questioned [40]. We thus cloned the human ACAD11 adding a Myc-tag at the N-terminus of the protein (Myc-*Hs*ACAD11). To investigate targeting of *Um*ACAD11n in mammalian cells, an N-terminally Myc-tagged fusion of um06400 was generated. COS-7 cells were transfected with these plasmids and with a Myc-tagged fusion of rat ACAD11 [32].

After 24 hours cells were processed for immunofluorescence using antibodies directed to the Myc epitope and the peroxisomal membrane marker Pex14p. All three fusion proteins were properly targeted to peroxisomes in COS-7 cells (**Fig. 3**) and co-localised with endogenous Pex14p. Peroxisomal targeting was also observed in HeLa and HepG2 cells (not shown). In addition, C-terminally tagged versions of all three ACAD11 proteins were generated (*Hs*ACAD11-GFP, *Rn*ACAD11-Myc, *Um*ACAD11n-Myc). However, expression in COS-7 cells did not result in peroxisomal targeting (**Suppl. Fig. S2**) due to blockage of the C-terminal PTS1 by the fused tag. The expressed proteins remained in the cytosol and no mis-targeting, e.g. to mitochondria, was observed.

To support these findings *in vivo*, the localization of ACAD11 was validated by immunoblotting of subcellular fractions isolated from rat liver. ACAD11 (~80 kDa) was strongly enriched in the peroxisome-containing fraction together with the peroxisomal marker proteins Pex14 and ACOX1, but of low abundance in mitochondria- or microsome-enriched fractions (**Fig. 3M**). The ACAD antibody also detected a more prominent band at ~60 kDa which may be indicative for an intraperoxisomal processing of ACAD11 by proteases (as reported for ACOX1), or the import of splice variants, which have been described recently [40] but may also result from degradation processes during organelle purification.

These findings clearly demonstrate that ACADs are *bona fide* peroxisomal proteins in fungi and mammals. Their peroxisomal targeting is conserved among species, and more importantly, they belong to the basic enzymatic repertoire of peroxisomes. As discussed later, a potential dehydrogenase-based fatty acid β-oxidation system in peroxisomes would offer the possibility to modulate peroxisomal H_2O_2 production by ACOX.

3.2 *U. maydis* has a complex enzymatic inventory for peroxisomal and mitochondrial fatty acid β-oxidation

Encouraged by our findings on ACADs, we decided to undertake a comprehensive characterisation of peroxisomes in *U. maydis*. The *U. maydis* genome was screened for proteins with a PTS1 or PTS2. As we observed non-canonical C-terminal PTS1 sequences (e.g., for ACADs), we initially searched with the broader consensus (ASCNPHTG)-(RKHQNS)-(LMIVF) [19]. All candidate proteins were thereafter analysed by PTS1 predictor algorithms and further validated by screening for conservation of the potential PTS1, and prediction of mitochondrial and ER targeting as well as presence of transmembrane domains. Our approach revealed a total of 124 candidates bearing a PTS1 or PTS2 (**Suppl. Table S3**). Functions were attributed with regard to their homology to known proteins from other species and proteins were organised into specific metabolic pathways partially supported by phylogenetic analyses (**Suppl. Table S3; Table 2**). In addition, peroxisomal membrane proteins and peroxins were identified by key word search and BLAST analysis.

To reveal if peroxisomes and mitochondria contribute to fatty acid β-oxidation in *U. maydis*, we first focused on key proteins and enzymes involved in fatty acid degradation. We identified candidate genes coding for all the enzymes required for a parallel peroxisomal and mitochondrial fatty acid β-oxidation in *U. maydis* (**Table 1; Suppl. Table S3; Fig. 4**) and performed a phylogenetic analysis to validate their conservation across species.

Prior to their degradation, fatty acids must be conjugated to coenzyme A and subsequently imported into peroxisomes by ABC class D transporters [41]. In *U. maydis* two transporters homologous to the yeast ABC half transporters PXA1 (um03945) and PXA2 (um01105) were identified (**Table 1, Suppl. Table S3; Fig. 4)**. Both membrane proteins are related to the mammalian peroxisomal transporters ABCD1 and ABCD2; a putative orthologue of mammalian ABCD3 was not detected in *U. maydis* but was found in the more basal Mucormycotina (**Suppl. Fig. S3**).

For the first step in peroxisomal β-oxidation catalysed by acyl-CoA oxidase (ACOX) four candidate genes were found: um01966, um02028, um02208 and um04324 (**Table 1, Suppl.**

Table S3; Fig. 4). All except um04324, the homologue of *Sc*POX1, possess a PTS1. Remarkably, after phylogenetic reconstruction we observed that this lack of a PTS is conserved among all fungi analysed (**Suppl. Fig. S1**) suggesting PTS-independent targeting mechanisms. *Sc*POX1 lacks a PTS1 but is still imported into peroxisomes in a Pex5-dependent manner without interacting with the PTS1-binding domain of Pex5 [42]. Similarly, in the yeast *Y. lipolytica* ACOX is imported into peroxisomes in a Pex5-dependent way in the form of a fully assembled heteropentamer, despite the absence of a PTS on any peptide chain [43]. It is likely, that besides POX1/um04324, more peroxisomal proteins exist, which follow a conserved Pex5-dependent but PTS1-independent import mechanism. Potential candidates identified in this study are the peroxisomal D-BP (um10038) which also lacks a PTS1 in several basidiomycetes and ascomycetes, and um02990 - a hydroxysteroid dehydrogenase-like protein which exhibits a conserved PTS1 among animals and other fungi (**Suppl. Table S3, Table 2**).

The second and third steps of peroxisomal β-oxidation are catalysed by two phylogenetically unrelated "multifunctional enzymes", the L- (L-BP) and the D-bifunctional protein (D-BP) [2] (**Fig. 4**): Unlike mammals, *U. maydis* (and all fungi investigated), harbours only a D-BP (um10038) whereas the L-BP was exclusively found in animals and the choanoflagellate *M. brevicolis* (**Table 1, Suppl. Table S3, Suppl. Figs. S4, S5**). Likewise, the L-BPs' close relative, the mitochondrial trifunctional enzyme subunit ECHA, is confined to the same eukaryote classes (**Suppl. Fig. S4**). However, other putative peroxisomal proteins were found in *U. maydis* which likely fulfil enzymatic reactions associated to the function of the multifunctional enzymes (**Table 1, Suppl. Table S3)**. These include several enoyl-CoA hydratases (um01747, um02097, um11001) and a 3-hydroxyacyl-CoA dehydrogenase of the SDR-family (um10825) which all possess a putative PTS1 (**Table 1, Suppl. Table S3)**. The fourth and last enzymatic reaction in β-oxidation is catalyzed by a 3-ketoacyl-CoA thiolase (**Fig. 4**). Two homologous genes were identified in *U. maydis*: um01090 with a predicted

PTS2, and um02715 without a PTS (**Table 1, Suppl. Table S3)**. Additionally, in mammals the peroxisomal isoform of the sterol carrier protein (SCP-X) is an alternative thiolase contributing to peroxisomal β-oxidation; two corresponding genes exist in *U. maydis* (um11938 with -AKL as PTS1, and um01986 without a PTS) but lack the name-giving SCP domain. As a consequence, we identified individual SCPs bearing a PTS1 in fungi including *U. maydis* (um11938, um01850) (**Table 1, Suppl. Table S3)**.

The mitochondrial fatty acid β-oxidation pathway starts with a dehydrogenation reaction catalysed by acyl-CoA dehydrogenases. Seven ACAD candidate genes were identified: um00694, um01049, um06185, um10665, um01466, um06400 and um00122 (**Table 1, Suppl. Table S3; Fig. 4**). The genes um01049 and um06185 correspond to the mammalian enzymes ACADM and ACDSB, whereas um00694 was only found in a few species in a patch-like pattern and resembles the prokaryotic FadE12 ACAD class (**Suppl. Fig. S1**). As outlined above, the latter four candidates possess a predicted C-terminal PTS1 suggesting peroxisomal localisation (**Table 1; Suppl. Table S3; Suppl. Fig. S1**), whereas two of the former three are predicted to be targeted to mitochondria.

For the second step in mitochondrial β-oxidation, commonly performed by the enoyl-CoA hydratase, three candidate genes (um01433, um02762, um11556) were identified, which were not predicted to be targeted to peroxisomes (**Table 1, Suppl. Table S3; Fig. 4**). Um11556 represents a homolog of human medium-chain enoyl-CoA hydratase; a deletion of its homolog in *Magnaporthe oryzae* has been shown to seriously affect mitochondrial β-oxidation [44]. For the third and fourth reaction of mitochondrial β-oxidation catalyzed by 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase two candidate genes were identified, respectively (um01099, um02105 and um03571, um03298) (**Table 1; Suppl. Table S3; Suppl. Fig. S6; Fig. 4**).

To experimentally confirm the existence of parallel fatty acid β-oxidation systems in peroxisomes and mitochondria in *U. maydis*, we determined the subcellular localization of

selected ACOX and ACADs by expression of fluorescent fusion proteins. An N-terminal GFP fusion of um02208, encoding for a putative ACOX, colocalised with mCherry-SKL thus confirming peroxisomal targeting in *U. maydis* (**Fig. 2G-I**). Likewise, an N-terminal mCherry fusion of the catalase homolog um11067, as a classic marker for the organelle, colocalized with GFP-SKL (**Fig. 2P-R**). In both cases, peroxisomes appear as spherical or rod-shaped organelles in *U. maydis*, which is reminiscent of their morphology in mammalian cells (**see Figs. 3, 5**).We further studied the localisation of the potential medium-chain acyl-CoA dehydrogenase (um01049, ACADM) and the short-branched chain acyl-CoA dehydrogenase (um06185, ACDSB). Both candidate enzymes for the first step in mitochondrial β-oxidation (**Table 1; Fig. 4**) were fused to mCherry at their C-termini and ectopically integrated into an *U. maydis* strain with GFP-labelled mitochondria (FB2 Lga-GFP). ACADM and ACDSB both co-localized with the *U. maydis*-specific mitochondrial matrix protein Lga2 (**Fig. 2J-O**), confirming targeting to mitochondria, which exhibited a tubular morphology. Thus, *U. maydis* shows the typical distribution of peroxisomal ACOX and mitochondrial ACADS found also in animals.

Our findings demonstrate that *U. maydis* possesses a complete and complex set of appropriate enzymes to carry out cooperative peroxisomal and mitochondrial fatty acid β-oxidation which resembles the situation in humans and animals.

3.3 Peroxisomes from *U. maydis* are responsive to fatty acid treatment

Mammals and yeast species respond to dietary fatty acids with a pronounced proliferation of peroxisomes and an induction of β-oxidation enzymes in order to elevate their capacity for lipid degradation [15]. To examine if and to what extend *U. maydis* responds to fatty acid treatment with a proliferation of peroxisomes, the strain FB2 GFP-SKL was incubated in minimal medium containing 0.2 % oleic acid as the sole carbon source as well as under glucose-containing control conditions. Peroxisome numbers per cell were determined by

fluorescence microscopy (**Fig. 5A, B**). In contrast to *S. cerevisiae*, but similar to the situation in mammalian cells many peroxisomes constitutively exist in *U. maydis* under non-induced growth conditions on glucose. Peroxisomes were observed to almost double already after 1 hour of oleic acid treatment (65.9±19.8, n=41) when compared to the appropriate controls (40.2±11.6, n=44) revealing a considerably sensitive response to fatty acids. Interestingly, oleic acid treatment induced the formation of elongated, rod-shaped peroxisomes and constricted, "bead on a string"-like morphologies which are reminiscent of processes of peroxisomal growth and division also observed in mammalian cells (**Fig. 5**) [45]. Peroxisomes in *U. maydis* are uniformly distributed over the cell and show dynamic, microtubule-based movements [26].

To investigate whether peroxisomal β-oxidation enzymes were induced in parallel to peroxisome proliferation, the expression level of peroxisomal ACOX (um02208) as a key enzyme in peroxisomal β-oxidation, was determined by a semi-quantitative RT-PCR approach using RNA from oleic acid and glucose-grown cells. Compared to controls, a significant increase in ACOX mRNA expression was observed after treatment with oleic acid (**Fig. 5C, D**). In contrast, expression of Elongation Factor 1 (EF1) or peptidyl-prolyl cis–trans isomerase (PPI), two non-peroxisomal house-keeping proteins, remained unaltered. As in animals, the induction of peroxisomal β-oxidation was accompanied by an increased expression of mitochondrial ACADM and ACDSB, pointing to cooperative function of both organelles in fungal lipid metabolism **(Fig. 5)**. These findings demonstrate that treatment with oleic acid induces a fast and pronounced peroxisome proliferation in *U. maydis* which is accompanied by up-regulation of ACOX. Its transcription is modulated by the carbon source, as predicted from its key function in peroxisomal β-oxidation. In yeast species, the transcription factors Oaf1p and Pip2p are known to regulate the fatty acid-induced elevation of β-oxidation enzymes during peroxisome proliferation. Other ascomycota, which do not possess homologues of these transcription factors, instead make use of the fungal transcription

factors farA/CTF1 and farB/CTF2 (see [15] for review). However, in basidiomycota our genome and phylogenetic analyses did not reveal the existence of either homologues and rather suggest a parallel evolution of a yet unidentified system of alternative transcription factors (data not shown).

3.4 *U. maydis* peroxisomes sequester a spectrum of fatty acids overlapping with mitochondrial β-oxidation

To investigate the contribution of peroxisomes and mitochondria to fatty acid metabolism in *U. maydis*, we generated a SG200 Δ*pex3* knockout strain. Deletion of PEX3, a peroxisomal membrane biogenesis factor, results in a complete loss of mature peroxisomes in all species investigated so far [46]. Hence, peroxisomal enzymes mislocalize to the cytosol and all associated enzymatic activities are profoundly reduced. A Δ*pex3* strain will thus predominantly rely on mitochondrial fatty acid β-oxidation when grown on fatty acids as sole carbon source. A complete loss of punctuate peroxisomal structures with a faint cytoplasmic accumulation of GFP-SKL was confirmed in the deletion strain by fluorescence microscopy (**Fig. 6A**), whereas reintroduction of PEX3 resulted in the re-appearance of peroxisomes (not shown). When cultivated on glucose the colony morphology of the Δ*pex3* strain was largely indistinguishable from the wild-type (**Fig. 6B**). However, the Δ*pex3* strain grows with slightly reduced rates when compared to the wild-type strain of *U. maydis* (**Fig. 6C**).

To analyze the spectrum of fatty acids degraded in peroxisomes, both the wild-type and Δ*pex3* strains were cultivated in glucose-free growth media supplemented with a broad spectrum of fatty acids in a concentration of 0.001% (**Fig. 6D**). The density and size of the individual colonies were taken as a measure for the ability to grow on the different carbon sources tested. To discriminate if growth reductions depend solely on the loss of peroxisomes, and thus the inability to break down fatty acids for energy generation, or if accumulating fatty

acids exert toxic effects on fungal growth, controls were performed in which the fatty acid media were supplemented with 1% glucose. As shown in **Fig. 6D** (- glucose), the Δ*pex3* mutant cells form less densely grown colonies on all lipid nutrients tested, whereas growth on acetate was not compromised. However, on saturated short- to medium-chain fatty acids (C4-C10), significant growth was still observed implying that breakdown of these fatty acids occurs also and maybe primarily in mitochondria and does not depend on peroxisomal β-oxidation. Growth on saturated long-chain fatty acids (myristic, palmitic acid) was found to be profoundly reduced in the Δ*pex3* mutant, while loss of peroxisomal β-oxidation vastly suppressed growth on very long-chain (lignoceric acid) and most unsaturated fatty acids (e.g., myristoleic, palmitoleic, oleic acid). Moreover, the latter fatty acids, when accumulating due to loss of peroxisomal function, exhibited significant toxicity (**Fig. 6D**). Even supplementation with glucose as an alternative energy source did not restore growth if compared to the wild-type strain (**Fig. 6D**, + glucose). Remarkably, palmitoleic acid exhibited the most severe toxicity. Interestingly, this fatty acid was recently described as a potent inducer of liponecrosis in the yeast *S. cerevisiae* [47]. These findings indicate that unsaturated fatty acids such as myristoleic, palmitoleic or oleic acid can only be degraded in significant amounts in peroxisomes in *U. maydis*. According to our genomic screen, *U. maydis* peroxisomes possess an incomplete enzyme inventory required for α-oxidation of C3-branched-chain fatty acids (**Table 2, Suppl. Table S3**). Nevertheless, peroxisome-deficient cells showed a near to zero growth rate, when phytanic acid was offered as sole carbon source while wild-type cells grew well. As an alternative to the α-oxidation pathway mammals are able to degrade 3-methyl branched-chain fatty acids in the ER via ω-oxidation using cytochrome P450 enzymes [48]. The resulting pristanic acid is subsequently degraded in peroxisomes via β-oxidation. Thus, *U. maydis* could utilize a similar combined ER/peroxisomal degradation system for 3-methyl branched-chain fatty acids, since the Δ-Pex3 strain was also unable to grow on pristanic acid. As *U. maydis*, in parallel to the

situation in mammals, houses a whole set of potential acyl-CoA oxidases and also a significant number of potential peroxisomal ACADs, individual enzymes have likely developed specialized substrate preferences, explaining the broad spectrum of fatty acids apparently sequestered in peroxisomes. In summary, our data demonstrate that *U. maydis* – similar to the situation in mammalian cells – utilizes both a peroxisomal and a mitochondrial β-oxidation pathway for fatty acid breakdown. Comparable to humans, *U. maydis* peroxisomes process preferentially longer chain and unsaturated fatty acids as well as branched-chain fatty acids, whereas short- and medium-chain fatty acids are (also) degraded in mitochondria. According to the growth experiments, however, the fungal peroxisomal β-oxidation pathway appears to handle a somewhat wider variety of fatty acids, resulting in a higher degree of overlap between mitochondrial and peroxisomal fatty acid specificity which may explain the total reduction of mitochondrial fatty acid β-oxidation in yeast species.

3.5 Comparison of the basic peroxisomal protein inventory shared by humans and fungi

Our comprehensive analysis revealed candidate proteins for several other important metabolic pathways in *U. maydis* peroxisomes (**Table 2**). Interestingly, a significantly larger overlap between the known mammalian peroxisomal proteome and homologous genes from *U. maydis* was observed when compared to the yeast *S. cerevisiae* (**Table 2**): several putative hydrogen-peroxide generating, flavin-linked oxidases (including many acyl CoA oxidases, urate oxidase, pipecolic acid oxidase, D-amino acid oxidase, polyamine oxidase) are shared but are absent in *S. cerevisiae* (**Table 2**). Furthermore, *U. maydis* orthologues for human proteins were identified which contribute to peroxisomal metabolism of ROS, amino acids, and purines (**Table 2, Suppl. Table S3**). Ether lipid synthesis (e.g. the synthesis of myelin sheath lipids and plasmalogens) is a characteristic feature of peroxisomes in animals, which requires metabolic cooperation with the ER. It is supposed to be absent in plants and yeast.

Interestingly, except for ADHAP, the key enzyme of peroxisomal ether lipid synthesis (which may not exist in fungi), we found homologs of all remaining proteins of the peroxisomal part of this pathway in *U. maydis* but not in *S. cerevisiae* (**Table 2**). The lack of an obvious ADHAP implies that the remaining enzymes may be involved in other lipid metabolic pathways such as the synthesis of glycerolipids and fatty alcohols, respectively. Thus only a single enzyme (ADAHPS) had to emerge in animal ancestors to complete this peroxisomal pathway. Furthermore, homologues of the human peroxisomal membrane proteins PXMP4, MOSC2/mARC, and ACBD5 appear to be present in *U. maydis* (**Table 2**). MOSC2/mARC and ACBD5 have been identified in recent proteomics studies of mammalian peroxisomes [32, 39, 49]. The former is involved in the reduction of N-hydroxylated drugs in mitochondria [50], but its peroxisomal function is unknown. A potential homologue of ACBD5, Atg37, has recently been linked to pexophagy in the yeast *P. pastoris* [51]. *U. maydis* encodes 21 of the 34 peroxisome biogenesis factors (peroxins, PEX) [52], which have been described in yeast, plants and animals (**Suppl. Tables S3, S4**). Among them are PEX16 (peroxisomal membrane biogenesis) and PEX26 (import receptor recycling), which are shared by *U. maydis* and *H. sapiens* but are absent in *S. cerevisiae*. Furthermore, two isoforms of PEX11 (um04294, um00440), which is associated with growth, division and proliferation of peroxisomes, were identified (**Suppl. Table S3, S4**). Multiple PEX11 genes are, in contrast to *S. cerevisiae,* typically found in plants and animals suggesting that a functional complementation by more than one PEX11 is a common strategy among most eukaryotes.

Our analysis also contributed to the identification of novel predicted peroxisomal proteins restricted to fungi. In addition to a couple of proteins with no predicted function, we identified an *U. maydis* protein (um01197) related to 8-amino-7-oxononanoate synthase, an enzyme involved in biotin synthesis with a canonical PTS1 (**Table 2, Suppl. Table S3**) – a pathway recently associated with peroxisomes in filamentous fungi [53]. Interestingly, metabolic coupling between peroxisomes and mitochondria appears to be required for biotin

biosynthesis. Furthermore, *U. maydis* gulonolactone oxidase (um10013), which is involved in ascorbate synthesis, and several enzymes with a function in carbohydrate metabolism (um00555, um10167, um11593) or mannosylerythritol lipid (MEL) biosynthesis (um03116, um10636) bear potential PTS1 sequences (**Table 2; Suppl. Table S3**). Peroxisomal targeting of the latter two acyltransferases Mac1 and Mac2 and involvement of peroxisomes in MEL biosynthesis in *U. maydis* has very recently been confirmed [54].

Among the *U. maydis* proteins with a predicted PTS1, we also revealed several proteins with unknown function (**Table 2, Suppl. Table S3**). Our sequence comparison to humans allowed us to identify some of them as potential homologues of known peroxisomal proteins (e.g., um10331 as peroxiredoxin V, um12269 as hydroxyl acid oxidase 1 or um06422 as the ACAD11 n-terminal APH domain) (**Suppl. Table S3**). Others are, however, confined to fungi or even Ustilaginomycetes and are likely to fulfil more species-specific functions. These species-specific proteins might reveal novel targets to combat pathogenicity of *U. maydis*.

Our studies revealed peroxisomal candidate proteins with unusual, non-canonical PTS1 sequences such as tripeptides PKL/PRL, SNL/SHL and GKL/GRL. Those were commonly identified among predicted *U. maydis* peroxisomal proteins and were often found on proteins associated with classical metabolic pathways of peroxisomes indicating functionality. In line with this, we demonstrated efficient peroxisomal targeting for the PTS1 –SHL (um06400, ACAD11n) (see **Fig. 2**). To investigate peroxisomal targeting of a predicted candidate protein with a weak PTS1, we choose Um11901. It is related to β-Lactamase-like protein 2 (LACTB2), a predicted zinc-binding hydrolase, which has been identified in recent proteomics studies of rat and human liver peroxisomes [32, 49]. Both the *U. maydis* and the rat homologue possess a predicted weak PTS1 (-SAL and –ASL, respectively). We thus investigated if the weak signal would be sufficient for targeting of LACTB2 to peroxisomes in *U. maydis* and mammalian cells. An N-terminal mCherry fusion of um11901 (mCherry-LACTB) did, however, not colocalise with GFP-SKL (or Lga-GFP) in *U. maydis* and resulted

in a cytosolic localisation (**Fig. 2S-U**). Thus, the predicted weak PTS1 sequences on uncharacterised candidate proteins (**Suppl. Table S3**) should be interpreted with care as they may not be functional. Interestingly, similar observations were made for Myc-*Rn*LACTB2 when expressed in COS-7 cells (**Fig. 3J-L**) and indicate that both proteins are either not peroxisomal or not efficiently targeted in our experimental setup. It should be noted that for the mouse homologue a mitochondrial localisation has been suggested [55]; however, proteins with both a peroxisomal and mitochondrial localisation are known [9].

4. Discussion

A primary role of peroxisomes is their contribution to cellular (and organismal) lipid homeostasis. Concordantly, fatty acid β-oxidation is a major metabolic pathway found in peroxisomes across species. Several genetic defects affecting the function of this pathway result in metabolic diseases [56]. In animals, peroxisomes cooperate with mitochondria which exhibit an alternative, more energy efficient β-oxidation system. In contrast, a mitochondrial β-oxidation pathway is absent in yeast and plants, which solely rely on peroxisomes for degradation of fatty acids.

Our comparative genetic analyses and localisation studies prove that *U. maydis* possesses a complex and complete enzymatic inventory for both peroxisomal and mitochondrial fatty acid β-oxidation, which resembles the situation in humans and animals. Notably, several other fungal species display only an incomplete set of mitochondrial β-oxidation enzymes [37], which renders *U. maydis* a suitable model system to study metabolic cooperation between peroxisomes and mitochondria. To unravel the overall peroxisomal contribution to fatty acid β-oxidation in *U. maydis*, we generated a mutant incapable of forming any peroxisomes by deletion of Pex3. According to the results obtained with the ΔPex3 deletion strain, mitochondrial β-oxidation in *U. maydis* is specific for saturated short to medium chain fatty

acids whereas peroxisomes sequester saturated and unsaturated fatty acids of a wide spectrum ranging from short to very long chain fatty acids. Previous studies using deletions of individual β-oxidation enzymes led to comparable results [58, 59]. Thus, the total deletion of peroxisomes proves that peroxisomal β-oxidation in *U. maydis* relies on the same set of enzymes as in animal cells. Since the Pex3-knockout shows a comparable inhibition of fatty acid β-oxidation as the single enzyme deletion strains mentioned above, it is unlikely that the additional proteins with homologies to β-oxidation enzymes found in our bioinformatics screen (um11001, um02097) contribute to this metabolic pathway. Rather, they are associated with other and potentially unknown peroxisomal functions.

The value of peroxisomal β-oxidation in *U. maydis* does not solely lie in energy production, but also serves detoxification purposes. We showed that the Δ*pex*3 mutant only grew poorly on unsaturated fatty acid media supplemented with glucose as an energy source. Such toxicity was also reported for *U. maydis* mutants with deletions in the Pex6 or D-PBE gene [59, 60]. The observed toxicity is likely caused by a disturbance of the ratio between saturated and unsaturated fatty acids in cellular membranes, thus compromising the biophysical properties of the endomembrane system [61].

Compared to animals, yeast species favour glucose, alcohol or organic acids for energy production, which could explain why fatty acid β-oxidation is entirely located to the less energy efficient peroxisomes and is only activated under conditions of acute shortage of other metabolites [62]. Indeed, in glucose-containing media, *S. cerevisiae* houses only around 1-2 peroxisomes per cell, whereas a change to fatty acids induces peroxisome numbers considerably. In *U. maydis* – similar to mammalian cells – many peroxisomes (40.2±11.6, n=44) are constitutively present suggesting that peroxisomes fulfil a more fundamental role in the fungus. In line with this, growth rates on glucose are slightly reduced for the Δ*pex3* strain. These findings indicate that similar to the situation in animals, peroxisomes of *U. maydis* are

required for additional important metabolic pathways, e.g., glycolysis [60] (see also our bioinformatics survey, **Suppl. Table S3**).

We demonstrated, that the *U. maydis* peroxisomal β-oxidation system responds to fatty acids by a coupled induction of the respective enzymatic inventory (e.g., ACOX) and an overall proliferation of the peroxisomal compartment. Like in mammals, peroxisome proliferation is accompanied by induction of the mitochondrial β-oxidation system, highlighting the alliance of both organelles in fatty acids metabolism. Apparently, even distantly related organisms such as basidiomycetes and humans retained ostensibly redundant enzyme inventories for a single metabolic β-oxidation pathway. Moreover, this scenario demands an associated regulation system between the two cooperating organelles. Thus, it is quite tempting to ask why such an - in terms of energetic efficiency - luxurious bimodal β-oxidation system was established?

The classic peroxisomal β-oxidation system consists of an ACOX which shows close homologies to acyl-CoA oxidases from δ-proteobacteria and high-GC Gram+ bacteria [63]. Subsequent steps in both animals and fungi are, however, performed by the bifunctional proteins (2^{nd}, 3^{rd} step) and 3-ketothiolase, which are more closely related to versions from α-proteobacteria suggesting a mitochondrial origin. Thus, the peroxisomal β-oxidation pathway appears to represent a hybrid of an ACOX derived from a primitive eukaryote and enzymes of mitochondrial origin, which were re-targeted to peroxisomes. Recently, Speijer [64] hypothesized, that the mitochondrial β-oxidation pathway for long chain fatty acids was transferred to peroxisomes to quench the formation of highly reactive oxygen species generated by the transfer of electrons from FADH through the OXPHOS complexes. In this regard the cooperation of mitochondria and peroxisomes in fatty acid β-oxidations mirrors a balance between energy effectiveness and toxicity of the by-products generated. Indeed, our phylogenetic analyses revealed a perpetual trend in *U. maydis* and in animals to relocate mitochondrial β-oxidation enzymes to peroxisomes.

In this respect, the identification of ACADs (ACAD11) as *bona fide* peroxisomal proteins in fungi and mammals is an important finding of this study. We clearly demonstrated that ACAD11 proteins from rat, *H. sapiens* and *U. maydis* are exclusively targeted to peroxisomes. In line with this, ACAD11 is highly enriched in peroxisome fractions isolated from rat liver. Our findings are further supported by recent proteomics studies of rodent and human peroxisomes [32, 39, 49] and the observation that human ACAD11 can interact with the peroxisomal PTS1 receptor Pex5 *in vitro* [65]. Our phylogenetic analyses revealed that ACAD11 proteins with peroxisomal targeting sequences exist in all eukaryotic phyla and belong to the basic enzyme repertoire of peroxisomes. This further indicates that peroxisomes and mitochondria in fungi and animals (with the exception of arthropods, where ACAD11 was apparently lost) both harbour enzymes of the acyl-CoA dehydrogenase family. ACAD11/10 homologues with a potential PTS1were also reported in plants [66] implying an ancient peroxisomal localisation of ACAD11 which was already established in the last common ancestor of all eukaryotes. In contrast, ACADs are completely absent in *S. cerevisiae* (which is known for loss of genes) [67]. Unlike in mammals and plants, *U. maydis* ACAD11 (and all other filamentous fungi) lacks an N-terminal APH domain. However, we identified a corresponding individual protein (um06422) in basidiomycetes as well as ascomycetes and confirmed its peroxisomal localisation. This observation does not support the hypothesis that the original ACAD11 in fungi was replaced by a truncated bacterial version via lateral gene transfer [66]. More likely, *U. maydis*, like most other fungi, possesses a split version of the mammalian ACAD11.

A fatty acid metabolizing activity of ACAD11 with highest affinities towards long- to very long-chain fatty acids (C20-C26) was experimentally determined [40] and shows overlap with the substrate spectrum of the peroxisomal acyl-CoA oxidase ACOX1. This prompts to ask about the function of peroxisomal ACADs in peroxisomal fatty acid β-oxidation.

A BLAST comparison of the sequence similarities of the ACAD11 dehydrogenase domain with those of bacteria suggests that it derived from α-proteobacteria and thus mitochondria. With respect to their enzymatic mechanism, peroxisomal ACOXs and mitochondrial ACADs function significantly different: ACOXs transfer the electrons liberated during the oxidation of the acyl-CoA via their FADH cofactor to molecular oxygen thus generating H_2O_2; in contrast, ACADs deliver the electrons from FADH to an electron acceptor protein (in mitochondria the electron transfer protein 1α, which further transfers the electrons to the respiratory chain) and do not form H_2O_2 but instead produce oxygen radicals during electron transfer through the mitochondrial electron transport chain.

It should be noted that our phylogenetic analyses revealed an additional potential ACAD with a PTS1 in fungi that possesses an N-terminal cytochrome b5-like domain (um01466). Similar cytochrome b5-enzyme fusions which are supposed to facilitate electron transfers have been described for other oxidoreductases [68, 69]. In this respect, it is remarkable that the ACAD11 N-terminal domain (ACADc) has a weak sequence similarity to the mitochondrial electron transfer flavoprotein α (ETFA). Thus, peroxisomal ACADs may be partially equipped with internal electron acceptor domains which in the case of the fungal ACAD11 are kept as individual protein entities; perhaps because there are further peroxisomal ACADs in fungi (um00122, um10665) which may have to interact with an electron acceptor protein.

β-Oxidation via peroxisomal ACOX generates toxic H_2O_2 which has to be removed by peroxisomal scavenging enzymes such as catalase. As H_2O_2 can also leak into the cytosol [70], the production of intra-peroxisomal H_2O_2 has to be carefully balanced. A potential dehydrogenase-based β-oxidation system in peroxisomes would offer the possibility to interfere with intra-organellar H_2O_2 production by regulation of the counteracting ACOX/ACAD pathways. Such a system could be fundamental to maintain peroxisomal H_2O_2 production at a controlled level, e.g., under conditions of enhanced fatty acid β-oxidation, or

to regulate the redox state inside (or in the periphery) of the organelle. H_2O_2 is also a potential signalling molecule which can catalyse the reversible formation of disulphide bridges [71]. In this respect, peroxisomes were reported to contribute to the H_2O_2-regulated control of neuronal activity [72] and to communicate with mitochondria modifying the redox potential of the latter [12, 13, 73]. Parallel ACAD/ACOX-based fatty acid β-oxidation in concert with catalase may build a complex regulation system for controlled release of H_2O_2 from peroxisomes in order to act as a cellular messenger. In summary, regulation of the production of ROS in mitochondria and peroxisomes appears to be one of the driving forces of the cooperative evolution of both organelles, switching enzymatic localisation and building a, still poorly-defined, signalling system to maintain the cellular redox potential at physiologically healthy levels. In this respect, *U. maydis* offers the potential to study such complex interrelationships in a model organism which is easy to cultivate and genetically accessible, but maintains the principle of organelle cooperation found in higher eukaryotes.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. ACAD11 is a conserved peroxisomal protein found across all opisthokonta.

(**A**) Simplified overview of the ACAD/ACOX phylogenetic distribution in opisthokont metazoa (original data presented in **Suppl. Fig. S1**). Both ACOX with peroxisomal and ACAD with mitochondrial targeting sequences are widely distributed in the kingdoms of animalia and fungi. Additional ACAD with a peroxisomal PTS1 are abundant in all opisthokont groups analysed (boxed). Only in Saccharomycetales, mitochondrial and peroxisomal ACAD were lost in a presumably step-wise process. IVD – Isovaleryl-CoA dehydrogenase, GCDH – Glutaryl-CoA dehydrogenase (**B**) The domain structure of ACAD11 in animals and choanoflagellata implies a fusion of two originally independent peroxisomal enzymes still separated in fungi (ACAD11n and ACAD11c).

Figure 2. Fatty acid β-oxidation enzymes localize to peroxisomes and mitochondria in *U. maydis*. Plasmids encoding GFP- or mCherry-labelled candidate fusion proteins (left column) were integrated ectopically into peroxisomal (AB33GFPSKL, FB1mChSKL) or mitochondrial (FB2LgaGFP) marker strains (middle column) and imaged live by laser-based epifluorescence microscopy in *U. maydis* yeast-like cells. Colocalisation of GFP-ACAD11n (um06400) (**A-C**) and

GFP-ACOX (um02208) (**G-I**) with the peroxisomal marker mCherry-SKL. (**D-F**) Colocalisation of ACAD11c-mCherry (um06422) with the peroxisomal marker GFP-SKL. The signals exhibit a punctuate pattern indicating peroxisomal localisation. Note that the halos result from peroxisomes which are not in focus. Peroxisomes in *U. maydis* are uniformly distributed over the cell and show microtubule-based movements. Colocalisation of ACADM-mCherry (um01049) (**J-L**) and ACDSB-mCherry (um06185) (**M-O**) with the mitochondrial

marker Lga2-GFP. The signals exhibit a tubular pattern indicating mitochondrial localisation. (**P-R**) Colocalisation of mCherry-CAT (um11067) with the peroxisomal marker GFP-SKL. (**S-U**) mCherry-LACTB (um11901) shows a cytosolic localization and does not colocalize with the peroxisomal marker GFP-SKL. Bar, 5 µm.

Figure 3. ACAD11 proteins from different species localise to peroxisomes in mammalian cells. COS-7 cells were transfected with plasmids encoding N-terminally tagged Myc-*Hs*ACAD11 (**A**), Myc-*Rn*ACAD11 (**D**), Myc-*Um*ACAD11n (**G**), or Myc-*Rn*β-Lact2 (**J**) and processed for immunofluorescence 24 h after transfection using antibodies directed to Myc (**A, D, G, J**) and the peroxisomal membrane marker Pex14 (**B, E, H, K**) and imaged by confocal microscopy. Note that ACAD11 proteins from *H. sapiens*, *R. norvegicus* and *U. maydis* are all exclusively targeted to peroxisomes. In contrast, β-Lactamase with a weak predicted PTS1 is not targeted to peroxisomes and remains cytosolic. Overlays are shown in (**C, F, I, L**). Nuclei are stained with DAPI. The corresponding C-terminally tagged ACAD11 fusion proteins show a cytosolic localisation (see **Suppl. Fig. S2**). Bars, 10 µm. (**M**) Immunoblot of subcellular fractions isolated from rat liver (10 µg of protein per lane). An antibody directed against rat ACAD11 (Abcam) detects ACAD11 (~ 80 kDa) and a 60 kDa band (asterisks), which are mostly prominent in peroxisome fractions (PO, purity > 95%, Islinger et al. 2007). Antibodies against the following proteins were used as organelle markers: Pex14, ACOX1 – peroxisomes; ATP synthase subunit α, VDAC1, ornithine carbamoyltransferase – mitochondria; GRP78, ERp29 – endoplasmic reticulum; tubulin α/β – cytosol. PNS – post nuclear supernatant, HM – heavy mitochondrial fraction, LM – light mitochondrial fraction (pre-fraction to PO), CYT – cytosolic fraction, MIC – microsomal fraction.

Figure 4. Comparison of peroxisomal and mitochondrial fatty acid β-oxidation pathways. β-Oxidation of fatty acids is carried out in peroxisomes and mitochondria in four subsequent reactions (① - ④), which are preceded by an activation step coupling the free fatty acids to coenzyme A. As long-chain fatty acids cannot pass the organelle membranes by mere diffusion, this has to occur outside the organelle involving membrane-bound long-chain acyl-CoA synthetases. The activated long-chain fatty acids are imported into the organelles by specific import systems, whereas short- and medium chain fatty acids are supposed to enter mitochondria by diffusion prior to activation. Concerning substrate specificity peroxisomes degrade long- to very long-chain fatty acids (> C16) down to a chain length of 8 carbon atoms. Octanoic acid is subsequently exported to mitochondria, which preferentially metabolize long- to short chain fatty acids. Mechanistic differences in fatty acid import and the catabolic reactions carried out in peroxisomes and mitochondria are marked in red; for all other steps both organelles possess individual sets of enzymes with substrate specificity towards different fatty acid chain lengths. Note that peroxisomes possess two bifunctional enzymes (D-BP, L-BP) combining steps ② and ③ in one protein. Mitochondria, however, can possess both, individual enzymes for each step of the pathway as well as a trifunctional enzyme consisting of two polypeptide chains (HadhA, HadhB) which combine steps ② to ④.

Figure 5. *U. maydis* peroxisomes are responsive to fatty acid treatment. *U. maydis* strain FB2 GFP-SKL was incubated in NM medium containing 1% glucose (control) or 0.2% oleic acid as the sole carbon source to induce peroxisome proliferation. (**A**) *U. maydis* yeast-like cells were imaged live by laser-based epifluorescence microscopy. Note the presence of elongated, rod-shaped peroxisomes and constricted “bead on a string”-like morphologies in cells treated with oleic acid, which resemble processes of peroxisomal growth and division in mammalian cells. DIC images are shown on the right. (**B**) Quantitative analysis of peroxisome

number per cell after 1 hour. Data are from 3 independent experiments (n= 44 cells) and are presented as mean ± SD. (**C**) To monitor the expression level of peroxisomal ACOX (um02208) and mitochondrial ACADM/ACDSB (um01049, um06185), total RNA was isolated from control and oleic-acid treated cells and RT-PCR was performed. The transcripts of the housekeeping proteins Elongation Factor 1 (EF1) and peptidyl-prolyl cis–trans isomerase (PPI) were amplified as loading controls. Triplicates of each condition are shown. Data are from 3 independent experiments and are presented as mean ± SD. Note induction of ACOX and ACAD expression in response to oleic acid exposure. (**D**) Representative gel images for the RT-PCR experiments.

Figure 6. Peroxisome deficiency and growth on fatty acids. (**A**) The peroxisomal marker GFP-SKL localizes to peroxisomes in the wild type Sg200 strain (upper panel), whereas GFP-SKL is cytosolic in the peroxisome-deficient Sg200 Δ*pex3* deletion strain. Note that exposure times have been increased to visualize cytosolic, non-peroxisomal GFP-SKL in the Δ*pex3* deletion mutant. DIC images are on the right. (**B**) Colony morphology of the wild type Sg200 and the Sg200 Δ*pex3* deletion strains on solid medium containing glucose. Light microscopy (left) and scanning electron microscopy (right) images. (**C**) Growth of the wild type Sg200 and the Sg200 Δ*pex3* deletion strains in CM liquid medium complemented with glucose. Data are from at least 3 independent experiments and are presented as mean ± SD. (**D**) The wild type strain Sg200 (WT) and the Sg200 Δ*pex3* deletion strain (KO) were spotted on solid medium containing different fatty acids (0.001%) or acetate (1%) in the absence or presence of glucose (1%). Photographs were taken after 48h at 28°C.

TABLES

Table 1. Fatty acid β-oxidation enzymes in *U. maydis*

Table 2. Inventory of *U. maydis* peroxisomal proteins and metabolic pathways in comparison to *H. sapiens* and *S. cerevisiae*

Supplementary material

Supplementary information

All identified proteins with a potential peroxisomal targeting signal 1 (PTS1) were analysed by PTS1 predictor algorithms [20] which evaluate the probability of targeting to peroxisomes by taking into consideration the C-terminal dodecamer [74]. This 12-amino acid sequence includes the most C-terminal tripeptide which is responsible for interaction with the surface of the Pex5p receptor-cavity, a tetrapeptide immediately upstream, thought to interact with the surface of the Pex5p proteins, and a flexible hinge that provides accessibility and flexibility. From this tool, query sequences that generate scores larger or equal to 0 are considered "predicted", sequences with scores below -10 are classified as "not predicted", and sequences with scores between 0 and -10 are considered being situated in a "twilight zone".

Suppl. Table S1. *Ustilago maydis* strains generated/used in this study

Suppl. Table S2. List of primers used in this study

Suppl. Table S3. *Ustilago maydis* theoretical peroxisomal protein inventory

Suppl. Table S4. Presence of peroxins in mammals, plants, filamentous fungi and yeast species

Suppl. Figure S1. Phylogram of acyl-CoA oxidase (ACOX) and acyl-CoA dehydrogenases (ACAD) catalyzing the first step in fatty acid β-oxidation. A phylogram of acyl-CoA oxidase and acyl-CoA dehydrogenases from > 30 fungal and animal species was generated with PhyML3.0 using the aLRT algorithm for branch support and including NNI

and SPR tree searching operations. Branches with a SH-aLRT branch support value above 0.75 are marked with a symbol (•). Black lines separate branches corresponding to the same enzyme category and are labeled to the right with the gene name of an already characterized member. If this was not possible, a short description of the proteins' common properties is given. The presence of organelle targeting sequences (PTS1/PTS2/MTS) identified by bioinformatics is indicated for each protein (no, not identified; twz, twilight zone). A group of potential ACADs with a facultative N-terminal cytochrome b5 domain (ACAD-Cytb5) was identified in fungi; the existence of the domain is additionally indicated for this branch of the tree.

Suppl. Figure S2. C-terminally tagged ACAD11 proteins from different species remain in the cytosol. COS-7 cells were transfected with plasmids encoding C-terminally tagged *Hs*ACAD11-GFP (**A**), *Rn*ACAD11-Myc (**D**), or *Um*ACAD11n-Myc (**G**), processed for immunofluorescence 24 h after transfection using antibodies directed to Myc (**D, G**) and the peroxisomal membrane marker Pex14 (**B, E, H**) and imaged by confocal microscopy. Note that tagging at the C-terminus abolishes peroxisomal targeting (see **Fig. 2**) and results in a cytosolic localization of ACAD11 proteins from *H. sapiens*, *R. norvegicus* and *U. maydis*. Overlays are shown in (**C, F, I**). Nuclei are stained with DAPI. Bars, 10 µm.

Suppl. Figure S3. Phylogram of ATP Binding Cassette (ABC) class D transporters. A phylogram of ABC class D transporters from > 40 fungal and animal species was generated and labeled as described in the legend to **Suppl. Fig. S1**. Branches with a SH-aLRT branch support value above 0.75 are marked with a symbol (•). Members of all families except for ABCD4 have been experimentally localized at peroxisomes and are supposed to import fatty-acyl-CoA for subsequent fatty acid β-oxidation.

Suppl. Figure S4. Phylogram of the enoyl-CoA hydratase enzyme family. Enoyl-CoA hydratases catalyze the second step of peroxisomal and mitochondrial fatty acid β-oxidation. Corresponding domains of the organelle-specific multifunctional enzymes were analyzed along with related single domain enzymes of the family. The phylogram was constructed from sequences of > 30 fungal and animal species and labeled as described in the legend to **Suppl. Fig. S1**. Branches with a SH-aLRT branch support value above 0.75 are marked with a symbol (•). The presence of organelle targeting sequences (PTS1/PTS2/MTS) identified by bioinformatics is indicated for each protein (no, not identified; twz, twilight zone). Note the neighboring of the branches comprising the enoyl-CoA domains of the peroxisomal L-BP (EHHADH) and the mitochondrial trifunctional enzyme subunit α (ECHA).

Suppl. Figure S5. Phylogram of the hydroxyacyl-CoA dehydrogenases involved in fatty acid β-oxidation. Hydroxyacyl-CoA dehydrogenases catalyze the third step of peroxisomal and mitochondrial fatty acid β-oxidation. Corresponding domains of the organelle specific multifunctional enzymes were analyzed along with selected single domain enzymes of the family. The phylogram was constructed from sequences of > 35 fungal and animal species and labeled as described in the legend to **Suppl. Fig. S1**. Branches with a SH-aLRT branch support value above 0.75 are marked with a symbol (•). The presence of organelle targeting sequences (PTS1/PTS2/MTS) identified by bioinformatics is indicated for each protein (no, not identified; twz, twilight zone). Note that the 17-beta-hydroxysteroid dehydrogenase 10 (HSD17B10) originally designed as an out-group to the peroxisomal D-BP (HSD17B4) was found to possess a predicted PTS1 in nearly all fungi analyzed.

Suppl. Figure S6. Phylogram of the 3-ketoacyl-CoA thiolases involved in fatty acid β-oxidation. 3-Ketoacyl-CoA thiolases/dehydrogenases catalyze the last step in peroxisomal and mitochondrial fatty acid β-oxidation. The phylogram was constructed from sequences of

> 30 fungal and animal species and labeled as described in the legend to **Suppl. Fig. S1**. Branches with a SH-aLRT branch support value above 0.75 are marked with a symbol (•). The presence of organelle targeting sequences (PTS1/PTS2/MTS) identified by bioinformatics is indicated for each protein (no, not identified; twz, twilight zone).